*INTRAMOLECULAR LOCALIZATION OF THE ACCEPTOR CROSS-LINKING SITES IN FIBRIN**

BY L. LORAND AND D. CHENOWETH

BIOCHEMISTRY DIVISION, DEPARTMENT OF CHEMISTRY, NORTHWESTERN UNIVERSITY, EVANSTON, ILLINOIS

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Abstract.—Using 1-^{14}C-glycine ethyl ester to titrate and label the acceptor cross-linking sites in fibrin, it was possible to localize and characterize the reactivity of these sites. In terms of sulfitolyzed chain fragments, both α and γ chains were shown to act as amine-acceptors, the site in γ being more reactive. Identification and isolation of the acceptor loci were also achieved after cyanogen bromide fragmentation. It is interesting that the "N-terminal disulfide knot" portion of fibrin does not seem to contain acceptor functions.

Cross-linking of fibrin constitutes the last enzymatic step in normal blood coagulation. In a transpeptidation reaction,[1-5] unique interfibrin γ-glutamyl-ϵ-lysine bonds form which strengthen the gel assembly. Without this process, the clot can be reversibly dissociated into the monomeric fibrin components in systems such as 30 per cent urea[6] or 1 per cent monochloroacetic acid.[7] The enzyme responsible for cross-linking is not present as such in plasma, but is produced—in limited proteolysis with thrombin[5, 8, 9] at the time of clotting—from its precursor (called fibrin stabilizing factor or Factor XIII).

The discovery that certain amines specifically inhibited the enzymatic cross-linking of fibrin[1, 4] without, however, interfering with aggregation (i.e., clotting) of the protein *per se* provided the key for elucidating the chemistry of the reaction. A number of these amines were shown to become incorporated by the cross-linking enzyme into the fibrin substrate. This was interpreted to signify that they would compete in the aminolysis of a fibrinyl-enzyme intermediate which, in the absence of the inhibitors, would react with the amino groups of another fibrin molecule. Hence, the amine inhibitors can be used to specifically titrate and to functionally modify the acceptor cross-linking sites in fibrin. This, of course, makes exploration of the latter possible. Glycine ethyl ester[10, 11] and histamine[12] were used as isotopic markers; hydroxylamine and hydrazine[2, 11] as chemical labels; and monodansylcadaverine[3, 4] [N-(5-aminopentyl)-5-dimethylamino-1-naphthalenesulfonamide] as a fluorescent tracer.

Concerning the identification of the immediate acceptor residue itself, analysis of the products of Lossen-rearranged fibrin hydroxamate provided proof that the enzymatically introduced label existed as the γ-glutamyl-hydroxamate.[2]

If it is assumed that fibrin has a molecular weight of 330,000 (i.e., 340,000† for fibrinogen[13] minus 3% allowance[15, 16] for the fibrinopeptides released in the formation of fibrin), the native protein appears to incorporate only approximately four moles of the amine. Kinetic analysis[3, 11, 17] shows that the first two equivalents are taken up rapidly and the other two more slowly (see also Fig. 1).

In terms of sulfitolyzed chain subunits,[18, 19] it has already been shown[3] with the

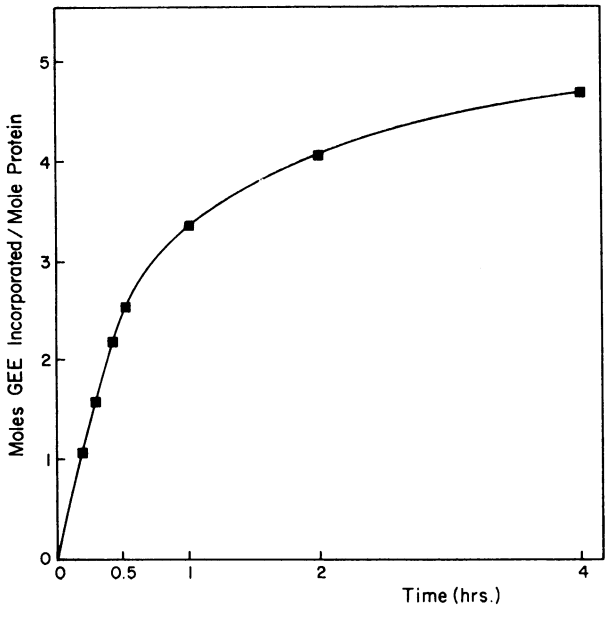

FIG. 1.—Time dependence of 1-^{14}C-glycine ethyl ester (*GEE*) incorporation into fibrin (a mol wt of 330,000 was used for the latter).

aid of the fluorescent monodansylcadaverine marker that the N-terminal tyrosine (or γ) chain carries the primary acceptors. Using 1-^{14}C-glycine ethyl ester labeling,[10, 11] we have now examined the intramolecular localization of amine acceptor sites in greater detail.

In the present paper, an identification of the acceptor loci will also be given in terms of the cyanogen bromide fragments of the protein.[20]

Materials and Methods.—1-^{14}C-glycine ethyl ester hydrochloride was purchased from New England Nuclear Corporation, Boston, Massachusetts.

Bovine fibrinogen was prepared from Armour Fraction I by the method of Laki.[21] It contained sufficient fibrin stabilizing factor as a contaminant so that addition of thrombin[22] caused both clotting and activation of the factor to the cross-linking transpeptidase.

Incorporation of 1-^{14}C-glycine ethyl ester into fibrin followed the general outlines given by Lorand and Jacobsen[10] and by Lorand and Ong.[11] The reaction was carried out at room temperature in a buffer containing 0.05 tris(hydroxymethyl)aminomethane and 0.1 *M* sodium chloride adjusted to pH 7.5 with hydrochloric acid. Concentrations of the reaction components were: fibrinogen 0.3%, calcium chloride 2 mM, cysteine 20 mM, glycine ethyl ester 20 mM (251 dpm μmole^{-1}), and finally thrombin 1.4 NIH units per ml. Incorporation of glycine ethyl ester was terminated at various times by the addition of an equal volume of 2% monochloroacetic acid, and the proteins were then precipitated by 7% trichloroacetic acid. Extractions with the latter continued until no more radioactivity could be removed. The proteins were finally washed with ethanol:ether (1:1 v/v) twice, then with ether, and dried.

About 5 mg of the protein was solubilized in 40% urea–0.2 *N* sodium hydroxide. Aliquots were taken for determining protein concentration[23] as well as for measuring radioactivity. For the latter purpose, 1 ml of protein solution was mixed with 12 ml of scintillation fluid.[24] Counting was carried out in a Packard liquid scintillation counter. Corrections were made both for counting efficiency and for quenching; the extent of incorporation was computed from a calibration curve relating glycine ethyl ester concentration to isotopic decay per minute.

Sulfitolysis of fibrin modified by glycine ethyl ester was carried out as recommended by Henschen.[19] The S-sulfo-chains of the protein were separated on a diethylaminoethyl-(DEAE)-Sephadex (A-50) column (2 × 20 cm), with a linear sodium chloride gradient[25] in 6 *M* urea and 0.03 *M* sodium acetate adjusted to pH 6.0 with acetic acid. Flow rate was set to 20 ml per hr at room temperature. Fractions of 4–5 ml were collected and analyzed both for ^{14}C radioactivity (by mixing 1-ml aliquots with 12 ml scintillation fluid) and for absorbancy at 280 mμ.

Cyanogen bromide fragmentation of labeled fibrin was performed according to the procedure of Blomback *et al.*[20] Peptides produced by this procedure were resolved on a Sephadex G-100 column (2.5 × 95 cm) equilibrated with 10% acetic acid. The column was eluted at a flow rate of 20 ml per hr at room temperature, fractions of 4–5 ml being collected. They were analyzed for absorbancy and radioactivity as above.

Results and Discussion.—It has been known since the quantitative examination of the N-terminal residues of bovine fibrin that the chemical unit consists of three types of open polypeptide chains,[26] one ending in tyrosine and two in glycine. The question as to which of these participate as acceptors in cross-linking could be simplified by asking which chains become labeled by the specific amine inhibitors of cross-linking. Clearly, this in turn can only be answered by analyzing a fibrin which, through the action of the cross-linking enzyme, had been maximally loaded with the tracer, 1-^{14}C-glycine ethyl ester in the present instance. Therefore, a sample corresponding to the modified fibrin at four hours of incor-

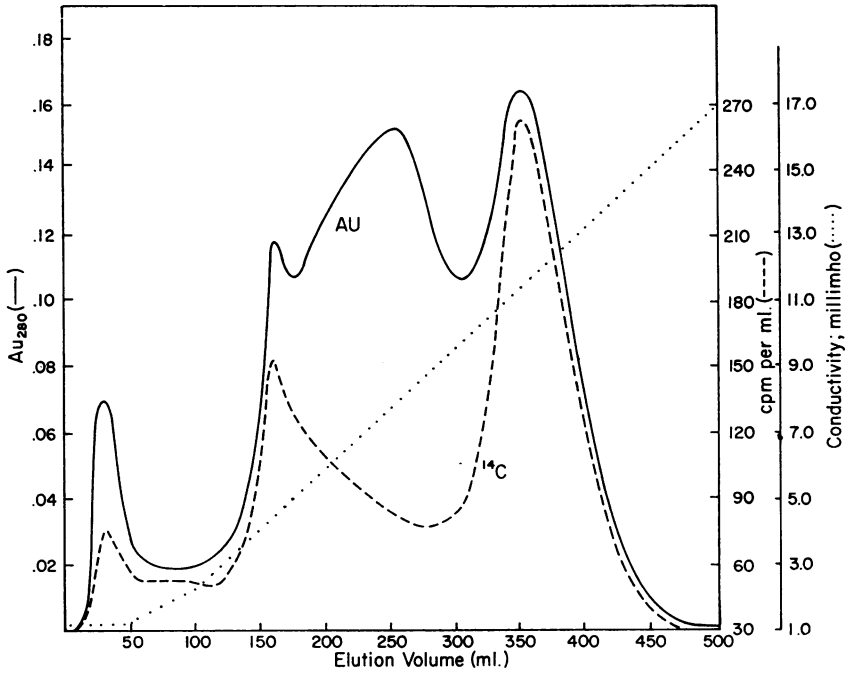

FIG. 2.—Separation of the S-sulfo-chains of fibrin modified with 1-^{14}C-glycine ethyl ester to the extent of 4.7 moles of GEE per mole protein (sample at 4 hr in Fig. 1) on DEAE-Sephadex. The peak eluting before the application of the salt gradient probably represents not fully sulfitolyzed protein.

AU, absorbancy units; *cpm*, ^{14}C counts per min.

poration (Fig. 1) was first examined. It is seen in Figure 2 that of the three chains of fibrin, only two, the so-called α or A and γ or C, contained the radioactive label with a roughly equal distribution of the isotope. The α chain (eluted between 3 and 5 millimho) is the one from which the fibrinopeptide A moiety was removed (carrying an N-terminus of glutamic acid in the bovine[15, 16] and alanine[27] in the human species) in the conversion of fibrinogen to fibrin by thrombin. The γ chain (eluted between 11 and 13 millimho) is unaffected by thrombin.

If, on the other hand, a fibrin modified to half the full extent (such as a sample in Fig. 1 stopped at 30 minutes) is similarly examined, isotope distribution reveals (Fig. 3) that γ chain labeling occurred almost exclusively. This finding is in agreement with earlier conclusions[3] using the fluorescent monodansylcadaverine label and CM-cellulose chromatography (or electrophoresis) for separating the sulfo-chains of fibrin.[18, 19]

As a preliminary to the exploration of primary sequences around the amine-acceptor sites, the cyanogen bromide fragments of modified fibrin were studied by Sephadex G-100 column chromatography according to the procedure of Blomback *et al.*[20] In order to gain insight into the relative order of filling of acceptor fragments obtained by this method of degradation, fibrin samples modified to varying extents with 1-^{14}C-glycine ethyl ester were again examined (Figs. 4 and 5). As seen in Figure 4, the peak eluting at about $V_e/V_t = 0.82$ seems to represent the fragment which carries the most reactive acceptor site.

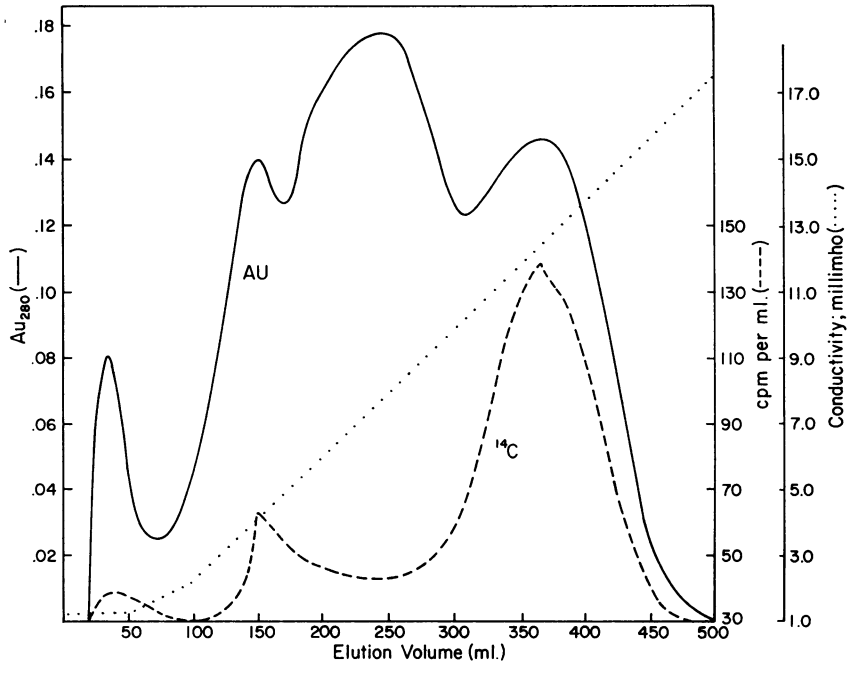

FIG. 3.—Separation of the S-sulfo-chains of fibrin only partially modified with 1-^{14}C-glycine ethyl ester (2.5 moles of the GEE per mole protein, the 0.5-hr sample in Fig. 1) on DEAE-Sephadex.

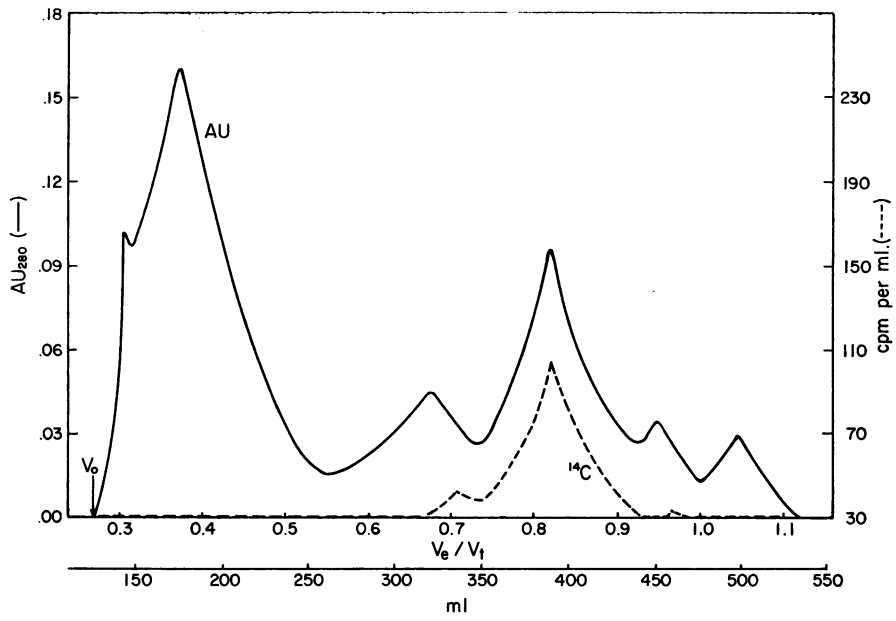

FIG. 4.—Sephadex G-100 column chromatography of fibrin fragmented by cyanogen bromide partially modified with 1-^{14}C-glycine ethyl ester (1.1 moles of GEE per mole protein, taken at 5 min, see Fig. 1).

V_0, void volume; V_e/V_t, elution volume divided by total column volume.

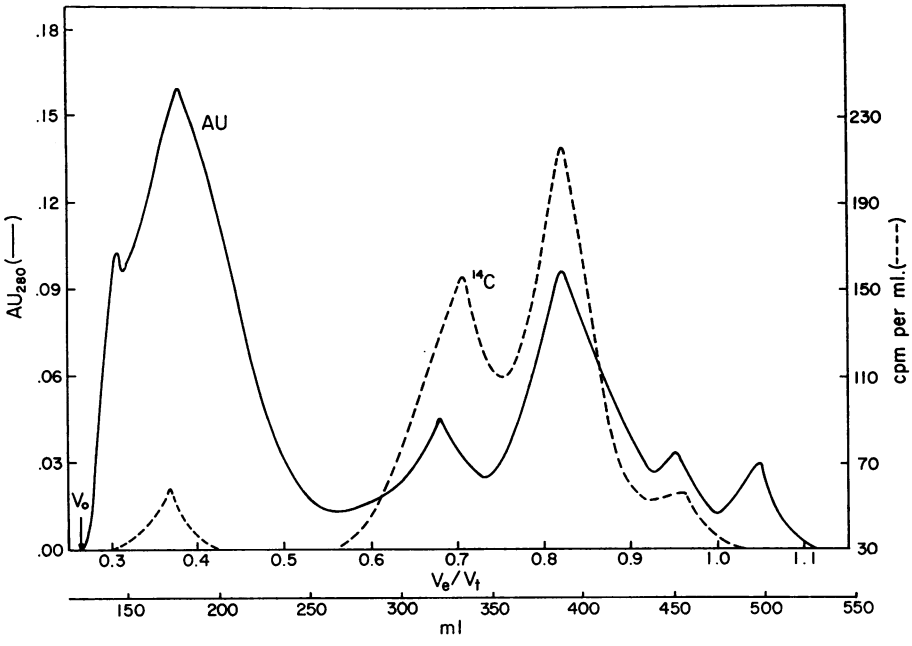

FIG. 5.—Sephadex G-100 column chromatography of cyanogen bromide fragmented fibrin completely modified with 1-^{14}C-glycine ethyl ester (4.7 moles of GEE per mole protein, the 4-hr sample in Fig. 1).

The relative absence of isotope from the larger fragments ($V_e/V_t \sim 0.3$–0.4) is quite conspicuous. The so-called "N-terminal disulfide knot" fragment (mol wt $\sim$ 26,000) of the protein would be eluted here,[20] still containing the end portions of all three chains linked by disulfide bridges. This fragment represents the region which is directly involved with thrombin in the fibrinogen-fibrin conversion. It is interesting to note that, in spite of the fact that removal of fibrinopeptide causes the unmasking of the amine-acceptor sites,[3, 11] no significant incorporation of the amine into this region of fibrin takes place.

In summary, both α and γ chains in fibrin were shown to contain amine-acceptor cross-linking sites, that in γ being more reactive. The amine acceptor groups of the protein were also identified and isolated in terms of cyanogen bromide fragments. The "N-terminal disulfide knot" portion of fibrin does not seem to contain acceptor functions.

Experiments with human fibrin gave identical results.

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† However, there is some evidence now[14] that the monomeric functional unit is only half as much. Should the molecular weight of fibrin be thus revised, only two amine labels per mole of protein would have to be considered, one of these incorporating into a rapidly reacting site, the other into a slower one.

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